इंटरनेट मानक

Disclosure to Promote the Right To Information

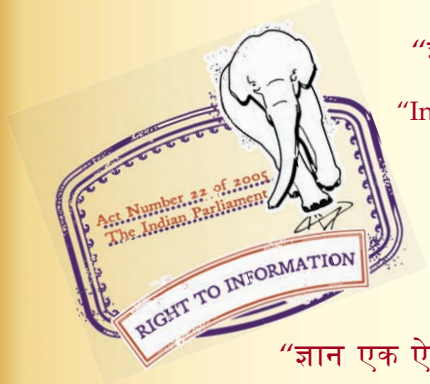
Whereas the Parliament of India has set out to provide a practical regime of right to information for citizens to secure access to information under the control of public authorities, in order to promote transparency and accountability in the working of every public authority, and whereas the attached publication of the Bureau of Indian Standards is of particular interest to the public, particularly disadvantaged communities and those engaged in the pursuit of education and knowledge, the attached public safety standard is made available to promote the timely dissemination of this information in an accurate manner to the public.

"जानने का अधिकार, जीने का अधिकार"
Mazdoor Kisan Shakti Sangathan
"The Right to Information, The Right to Live"

"पुराने को छोड नये के तरफ"
Jawaharlal Nehru
"Step Out From the Old to the New"

IS 13427 (1992): Animal feeds and feeding stuffs - Determination of aflatoxin B-1 content [FAD 5: Livestock Feeds, Equipment and Systems]

"ज्ञान से एक नये भारत का निर्माण"
Satyanarayan Gangaram Pitroda
"Invent a New India Using Knowledge"





"ज्ञान एक ऐसा खजाना है जो कभी चुराया नहीं जा सकता है"
Bhartṛhari—Nītiśatakam
"Knowledge is such a treasure which cannot be stolen"

BLANK PAGE





PROTECTED BY COPYRIGHT

IS 13427 : 1992
ISO 6651 : 1987

भारतीय मानक

पशु आहार और आहार सामग्री – एफलाटॉक्सिन बी$_1$ अंश ज्ञात करना

Indian Standard

ANIMAL FEEDS AND FEEDING STUFFS – DETERMINATION OF AFLATOXIN B_1 CONTENT

UDC 636·085 : 543 [516·9]

© BIS 1992

BUREAU OF INDIAN STANDARDS
MANAK BHAVAN, 9 BAHADUR SHAH ZAFAR MARG
NEW DELHI 110002

July 1992

Price Group 5

AMENDMENT NO. 1 AUGUST 1993
TO
IS 13427 : 1992 / ISO 6651 : 1987 ANIMAL FEEDS AND FEEDING STUFFS — DETERMINATION OF AFLATOXIN B_1 CONTENT

(*Page* 8, *clause* **9.2**) — Add the following note under clause **9.2** :

'NOTE — In the Indian context the collaborative study on the same feed samples between laboratories have indicated the variation of:

a) 50% of the mean value for mean value of aflatoxin B_1 between 10 to 20 ppb, and

b) 30% of mean value for the mean value of aflatoxin B_1 above 20 ppb.'

(FAD 5)

Reprography Unit, BIS, New Delhi, India

IS 13427 : 1992
ISO 6651 : 1987

Indian Standard

ANIMAL FEEDS AND FEEDING STUFFS – DETERMINATION OF AFLATOXIN B_1 CONTENT

NATIONAL FOREWORD

This Indian Standard which is identical with ISO 6651 : 1987 'Animal feeding stuffs — Determination of aflatoxin B_1 content', issued by the International Organization for Standardization (ISO), was adopted by the Bureau of Indian Standards on the recommendation of the Livestock Feeds Sectional Committee (FAD 5) and approval of the Food and Agriculture Division Council.

In the adopted Indian Standard, some terminology and certain conventions are not identical with those used in Indian Standards. Attention is especially drawn to the following:

a) Wherever the words 'International Standard' appear, referring to this standard, they should be read as 'Indian Standard'.

b) Comma (,) has been used as a decimal marker, while in Indian Standards, the current practice is to use point (.) as the decimal marker.

CROSS REFERENCE

In this Indian Standard, the following International Standard is referred to:

ISO 6498 : 1983 Animal feeding stuffs — Preparation of test sample.

The Technical Committee has reviewed the provisions of ISO 6498 : 1983 'Animal feeding stuffs — Preparation of test sample' to which reference is made in the text and has decided that this standard is acceptable for use in conjunction with this standard.

ADDITIONAL INFORMATION

The standard prescribes two methods:

Method A One dimensional thin layer chromatography, and

Method B Two dimensional thin layer chromatography.

The extraction procedure is the same for both these methods.

Method A is recommended for straight feeding stuffs and raw materials.

Method B is recommended for compounded feeds such as poultry and cattle feeds and also for raw materials such as cottonseed and its oilcakes and deoiled meals.

The following Note may be included under clause **5.6**:

'NOTE — The solvents, namely, diethyl ether (**5.3**) and chloroform (**5.5**) should be made free from traces of moisture by shaking with anhydrous sodium sulphate'.

The following may be included as an alternative developing solvent for Methods A and B in place of chloroform/acetone (90+10) if silica gel used is of Indian make:

'**5.6.6** Toluene/amyl alcohol/methanol (90 : 32 : 2).'

Include **5.6.6** as an alternate for choice of solvent under clause **8.5.1.1**.

Sampling of the product for aflatoxin analysis shall be done in accordance with IS 13426 : 1992 'Animal feeds and feeding stuffs — Methods of sampling for aflatoxin analysis'.

For the purpose of deciding whether a particular requirement of this standard is complied with, the final value, observed or calculated, expressing the result of a text or analysis, shall be rounded off in accordance with IS 2 : 1960 'Rules for rounding off numerical values (*revised*)'. The number of significant places retained in the rounded off value should be the same as that of the specified value in this standard.

1 Scope

This International Standard specifies two methods for the determination of the aflatoxin B_1 content of animal feeding stuffs.

2 Field of application

2.1 Method A is applicable to the following simple feeding stuffs :

- oilseeds and oilseed residues, and in particular groundnut, copra, linseed, soya, sesame, babassu palm;
- manioc meal;
- maize germ expeller;
- cereals and cereal products;
- pea meal;
- potato pulp and flour.

In the presence of substances interfering with the determination by method A, it is recommended that the determination be carried out in accordance with method B.

2.2 Method B is applicable to mixed feeding stuffs and to simple feeding stuffs not mentioned in 2.1.

This method is not applicable to feeding stuffs containing citrus pulp.

2.3 The lower limit of detection of aflatoxin B_1 is 0,01 mg/kg.

3 Reference

ISO 6498, *Animal feeding stuffs — Preparation of test samples.*

4 Principle

Extraction of the test portion with chloroform, filtration, and purification of an aliquot portion on a silica gel column. Evaporation of the eluate and dissolution of the residue in a specified volume of chloroform or mixture of benzene and acetonitrile.

Thin-layer chromatography, one-dimensional for method A and two-dimensional for method B, of an aliquot portion of this solution.

Determination of the aflatoxin B_1 content, either visually or by fluorodensitometry, by examination of the chromatogram under ultraviolet light and comparison with known quantities of standard aflatoxin B_1 applied to the same plate as the test portion extract.

Confirmation of the identity of aflatoxin B_1 by formation of the hemiacetal derivative.

5 Reagents

All reagents shall be of recognized analytical quality. The water used shall be distilled water or water of at least equivalent purity.

5.1 Chloroform, stabilized with 0,5 to 1,0 % of 96 % (*V*/*V*) ethanol.

5.2 ***n*-Hexane.**

5.3 Diethyl ether, anhydrous, free from peroxides.

5.4 Benzene/acetonitrile, (98 + 2) mixture.

Mix 98 volumes of benzene with 2 volumes of acetonitrile.

5.5 Chloroform/methanol, (97 + 3) mixture.

Mix 97 volumes of chloroform with 3 volumes of methanol.

5.6 Developing solvents.[1)]

5.6.1 Chloroform/acetone, (90 + 10) mixture.

Mix 90 volumes of chloroform with 10 volumes of acetone, in an unsaturated tank.

1) The solvents should be used in covered tanks. When saturated tanks are specified, this is achieved by lining the tanks with absorbent paper and allowing the interiors to become saturated with solvent vapour.

5.6.2 Diethyl ether/methanol/water, (96 + 3 + 1) mixture.

Mix 96 volumes of diethyl ether, 3 volumes of methanol and 1 volume of water, in an unsaturated tank.

5.6.3 Diethyl ether/methanol/water, (94 + 4,5 + 1,5) mixture.

Mix 94 volumes of diethyl ether with 4,5 volumes of methanol and 1,5 volumes of water, in a saturated tank.

5.6.4 Chloroform/methanol, (94 + 6) mixture.

Mix 94 volumes of chloroform with 6 volumes of methanol, in a saturated tank.

5.6.5 Chloroform/methanol, (97 + 3) mixture.

Mix 97 volumes of chloroform with 3 volumes of methanol, in a saturated tank.

5.7 Silica gel, for column chromatography, of particle size 0,05 to 0,20 mm.

5.8 Silica gel, G-HR or equivalent, for thin-layer chromatography.

5.9 Diatomaceous earth (Hyflosupercel), acid-washed.

5.10 Sodium sulfate, anhydrous granules.

5.11 Trifluoroacetic acid.

5.12 Inert gas, for example nitrogen.

5.13 Sulfuric acid, 50 % (*V*/*V*) solution.

5.14 Aflatoxin B_1, standard solution containing about 0,1 µg of aflatoxin B_1 per millilitre, in the chloroform (5.1) or in the benzene/acetonitrile mixture (5.4).

WARNING — Aflatoxins are highly carcinogenic and must be handled with great care.

Prepare and check the solution as follows.

5.14.1 Preparation of stock solution and determination of concentration

Prepare a solution of aflatoxin B_1 in the chloroform (5.1) or the benzene/acetonitrile mixture (5.4) such that the concentration is between 8 and 10 µg/ml. Determine the absorption spectrum between 330 and 370 nm by means of the spectrophotometer (6.9).

Measure the absorbance (A) at 363 nm in the case of the chloroform solution, or at 348 nm in the case of the benzene/acetonitrile mixture solution.

Calculate the concentration of aflatoxin B_1, in micrograms per millilitre of solution, from the formulae :

a) for the chloroform solution :

$$\frac{312 \times A \times 1\,000}{22\,300}$$

b) for the solution in the benzene/acetonitrile mixture :

$$\frac{312 \times A \times 1\,000}{19\,800}$$

5.14.2 Dilution

Dilute the stock solution (5.14.1), as appropriate, away from daylight, to obtain a standard solution with a concentration of aflatoxin B_1 of about 0,1 µg/ml.

If kept in a refrigerator at 4 °C, this solution is stable for 2 weeks.

5.14.3 Testing of chromatographic purity of the solution

Onto a plate (6.7), apply a spot of 5 µl of the standard aflatoxin B_1 solution of concentration 8 to 10 µg/ml (5.14.1). Develop the chromatogram as indicated in 8.5.1. Under ultraviolet light, the chromatogram shall show only one spot and no fluorescence shall be perceptible in the original deposition zone.

5.15 Aflatoxin B_1 and B_2 (see the warning in 5.14), solutions for qualitative testing, containing about 0,1 µg of aflatoxin B_1 and B_2 per millilitre, in the chloroform (5.1) or in the benzene/acetonitrile mixture (5.4).

These concentrations are given as a guide. They shall be adjusted so as to obtain the same intensity of fluorescence for both aflatoxins (see 8.5.1).

6 Apparatus

Usual laboratory equipment, and in particular :

6.1 Grinder/mixer.

6.2 Sieve, of aperture size 1,0 mm.[1)]

6.3 Shaking apparatus or **magnetic stirrer.**

1) See ISO 565, *Test sieves — Woven metal wire cloth, perforated plate and electroformed sheet — Nominal sizes of openings.*

6.4 Chromatographic tubes, made of glass (internal diameter 22 mm, length 300 mm), with a polytetrafluoroethylene tap and a 250 ml reservoir, plugged at the bottom end with cotton or glass wool.

6.5 Rotary vacuum evaporator, with a 500 ml round-bottomed flask.

6.6 Apparatus for thin-layer chromatography (TLC), i.e. that necessary for the preparation of the plates (6.7) and application of spots (capillary pipettes or microsyringes), a developing tank, and spraying apparatus for applying the sulfuric acid (5.13) to the plates.

6.7 Glass TLC plates, 200 mm × 200 mm, prepared as follows (the quantities indicated are sufficient to cover five plates).

Place 30 g of the silica gel (5.8) in a conical flask, add 60 ml of water, stopper and shake for 1 min. Spread the suspension on the plates so as to obtain a uniform layer 0,25 mm thick. Leave to dry in the air and then store in a desiccator containing silica gel. At the time of use, activate the plates by keeping them in an oven at 110 °C for 1 h.

Ready-to-use plates are suitable if they give results similar to those obtained with the plates prepared as indicated above.

6.8 Long-wavelength (360 nm) ultraviolet lamp.

The intensity of irradiation shall make it possible for a spot of 1,0 ng of aflatoxin B_1 to be clearly distinguished on a TLC plate at a distance of 10 cm from the lamp.

WARNING — Ultraviolet light is dangerous to the eyes. Protective goggles shall be worn.

6.9 Spectrophotometer, suitable for making measurements in the ultraviolet region of the spectrum.

6.10 Fluorodensitometer (optional).

6.11 Fluted filter paper.

6.12 Graduated tube, of capacity 10,0 ml, with a polyethylene stopper.

6.13 Conical flask, of capacity 500 ml, with a ground glass stopper.

6.14 Pipette, of capacity 50 ml.

6.15 Balance.

7 Sampling

Take the laboratory sample from the material to be sampled in accordance with the International Standard for the material concerned unless sampling for the determination of aflatoxin is excluded from its field of application. If no appropriate International Standard exists, agreement shall be reached between the parties concerned, taking into account the characteristics of the material being sampled.

8 Procedure

8.1 Preparation of the test sample

8.1.1 If the sample contains more than 5 % of fat, it shall be defatted with light petroleum before grinding.

In such cases, the analytical results shall be expressed in terms of the mass of the non-defatted sample.

8.1.2 Grind the laboratory sample so that it completely passes through the sieve (6.2). Mix thoroughly. (See ISO 6498.)

8.2 Test portion

Weigh, to the nearest 0,01 g, 50 g of the prepared test sample into the conical flask (6.13).

8.3 Extraction

Add to the test portion (8.2) 25 g of the diatomaceous earth (5.9), 25 ml of water, and 250 ml of the chloroform (5.1) accurately measured from a measuring cylinder. Stopper the flask, and shake or stir for 30 min using the apparatus (6.3). Filter through the fluted filter paper (6.11), taking care to discard the first 10 ml of the filtrate, and subsequently collect at least 50 ml of the filtrate.

8.4 Column clean-up

8.4.1 Preparation of the column

Fill two-thirds of the chromatographic tube (6.4) with the chloroform (5.1) and add 5 g of the sodium sulfate (5.10). Check that the upper surface of the sodium sulfate layer is flat, then add 10 g, in small portions, of the silica gel (5.7). Stir carefully after each addition to eliminate air bubbles. Leave to stand for 15 min and then carefully add 10 g of the sodium sulfate (5.10). Open the tap and allow the liquid to flow until it is just above the upper surface of the sodium sulfate layer. Close the tap.

8.4.2 Purification

Transfer, by means of the pipette (6.14), 50 ml of the filtrate collected in 8.3 to a 250 ml conical flask, and add 100 ml of the *n*-hexane (5.2). Mix and quantitatively transfer the mixture to the column, rinsing the flask with the *n*-hexane. Open the tap and allow the liquid to flow at a rate of 8 to 12 ml/min until it is level with the upper surface of the sodium sulfate layer. Close the tap. Discard the liquid collected and pour 100 ml of the diethyl ether (5.3) into the column. Again open the tap and allow the liquid to flow until it is level with the upper surface of the sodium sulfate layer. During these operations, ensure that the column does not run dry.

Elute with 150 ml of the chloroform/methanol mixture (5.5) and collect the whole of the eluate in the 500 ml flask of the rotary evaporator (6.5). Evaporate to dryness on the rotary evaporator, preferably under a stream of inert gas (5.12), at a temperature not exceeding 50 °C, and under reduced pressure.

NOTE – If a rotary evaporator is not available, add a boiling aid and evaporate almost to dryness on a water bath.

Quantitatively transfer the residue, using the chloroform (5.1) or the benzene/acetonitrile mixture (5.4), to the 10 ml graduated tube (6.12). Again evaporate the solution, for example in a water bath, preferably under a stream of inert gas (5.12), and adjust the volume to 2,0 ml with the chloroform (5.1) or the benzene/acetonitrile mixture (5.4).

8.5 Thin-layer chromatography

8.5.1 Method A – One-dimensional thin-layer chromatography

8.5.1.1 Choice of solvent

The choice of solvent (5.6.1, 5.6.2, 5.6.3, 5.6.4 or 5.6.5) shall be made beforehand to ensure that aflatoxins B_1 and B_2 are completely separated when the plate is developed, which depends on the batch of plates in use.

Place 25 µl of the qualitative solution (5.15) on the prepared plates (6.7) (one plate for each solvent to be checked).

Follow the procedure in 8.5.1.2 for development, evaporation and irradiation.

Two distinct spots are produced by a suitable solvent.

8.5.1.2 Procedure

Onto a TLC plate (6.7), and using a capillary pipette or microsyringe, apply 20 mm from the lower edge, and at intervals of 20 mm, the volumes indicated below of the standard aflatoxin B_1 solution and the extract :

– 10, 15, 20, 30 and 40 µl of the standard aflatoxin B_1 solution (5.14);

– 10 µl of the extract obtained in 8.4.2 and, superimposed on the same point, 20 µl of the standard aflatoxin B_1 solution (5.14);

– 10 and 20 µl of the extract obtained in 8.4.2.

Develop the chromatogram in the dark using the developing solvent chosen (see 8.5.1.1).

Remove the plate from the tank, allow the solvents to evaporate from the plate in the dark and then examine under ultraviolet light, placing the plate 10 cm from the lamp (6.8). The spots of aflatoxin B_1 show a blue fluorescence.

8.5.2 Method B – Two-dimensional thin-layer chromatography

8.5.2.1 Application of the solutions (see figure 1)

Trace two straight lines on a plate (6.7) parallel to two contiguous sides (50 and 60 mm from each side respectively), to establish the limit of migration of the solvent fronts. Apply the following solutions on the plate using capillary pipettes or microsyringes :

– at point A : 20 µl of the purified sample extract obtained in 8.4.2;

– at point B : 20 µl of the standard aflatoxin B_1 solution (5.14);

– at point C : 10 µl of the standard aflatoxin B_1 solution (5.14);

– at point D : 20 µl of the standard aflatoxin B_1 solution (5.14);

– at point E : 40 µl of the standard aflatoxin B_1 solution (5.14).

Dry in a slow stream of air or inert gas (5.12). The spots obtained shall have a diameter of about 5 mm.

8.5.2.2 Development (see figure 1)

Develop the chromatogram in direction I, in the dark, using the developing solvent (5.6.3) (1 cm layer in a saturated tank) until the solvent front reaches the limit line. Remove the plate from the tank and allow to dry, in the dark, at ambient temperature for at least 15 min.

Then develop the chromatogram in direction II, in the dark, using the developing solvent (5.6.1) (1 cm layer in an unsaturated tank) until the solvent front reaches the limit line. Remove the plate from the tank and allow to dry, in the dark, at ambient temperature.

8.5.2.3 Interpretation of the chromatogram (see figure 2)

Examine the chromatogram under ultraviolet light by placing the plate 10 cm from the lamp (6.8). Locate the position of the blue fluorescent spots B, C, D and E of the aflatoxin B_1 from the standard solution and trace two imaginary lines passing through these spots and at right angles to the directions of development. The point P of intersection of these lines is the location at which to expect the aflatoxin B_1 spot originating from the test portion extract applied at point A (see figure 1). However, the actual location of the aflatoxin B_1 spot may be at a point Q at the intersection of two imaginary straight lines forming an angle of about 100° between them and passing through points B and C respectively.

8.5.2.4 Supplementary chromatography

Trace on a new plate (6.7) two straight lines parallel to two contiguous sides, as indicated in figure 1, and apply, at point A, 20 µl of the purified test portion extract obtained in 8.4.2 and,

superimposed on it, 20 µl of the standard aflatoxin B_1 solution (5.14). Develop as indicated in 8.5.2.2. Examine the chromatogram under ultraviolet light and check that

— the aflatoxin B_1 spots from the extract and the standard solution are superimposed;

— the fluorescence of this spot is more intense than that of the aflatoxin B_1 spot developed at point Q on the first plate.

8.6 Determination

Two methods of determination may be used, i.e. visual measurement or fluorodensitometric measurement.

8.6.1 Visual measurement

8.6.1.1 Method A

Determine the quantity of aflatoxin B_1 in the extract by comparing the intensity of fluorescence of the extract spots with that of the standard solution spots. Interpolate if necessary.

The fluorescence obtained by the superimposition of the extract on the standard solution shall be more intense than that of the 10 µl of extract and shall be perceptible as only one spot. If the intensity of fluorescence given by the 10 µl of extract is greater than that of the 40 µl of standard solution, dilute the extract 10 or 100 times with the chloroform (5.1) or with the benzene/acetonitrile mixture (5.4) before repeating thin-layer chromatography.

8.6.1.2 Method B

Determine the quantity of aflatoxin B_1 in the extract by comparing the intensity of the extract spot with that of spots C, D and E from the standard solution. Interpolate if necessary.

If the intensity of fluorescence given by the 20 µl of extract is greater than that of the 40 µl of standard solution, dilute the extract 10 or 100 times with the chloroform (5.1) or with the benzene/acetonitrile mixture (5.4) before repeating thin-layer chromatography.

8.6.2 Measurement by fluorodensitometry

Measure the intensity of fluorescence of the aflatoxin B_1 spots with the fluorodensitometer (6.10) at an excitation wavelength of 365 nm and an emission wavelength of 443 nm.

Determine, in the case of method A, the quantity of aflatoxin B_1 in the extract spots by comparison with the intensity of fluorescence of the spots from the standard solution, and, in the case of method B, the quantity of aflatoxin B_1 in the extract spot by comparison with the intensity of fluorescence of spots C, D and E from the standard solution.

8.7 Confirmation of the identity of aflatoxin B_1

Confirm the identity of the aflatoxin B_1 in the extract by the presumptive test with sulfuric acid (see 8.7.1) and, if the result of this test is positive, by the actual confirmation test (8.7.2). If the result of the presumptive test with sulfuric acid is negative, there is no need to proceed with the actual confirmation since, in this case, no aflatoxin B_1 is present.

8.7.1 Presumptive test with sulfuric acid

Spray the sulfuric acid (5.13) on to the chromatogram obtained in 8.5.1 or 8.5.2. The fluorescence of the aflatoxin B_1 spots shall turn from blue to yellow under ultraviolet light.

8.7.2 Confirmation test : formation of aflatoxin B_1-hemiacetal (aflatoxin B_{2a})

In the case of simple and only slightly pigmented feeds, use the one-dimensional thin-layer chromatographic method described in 8.7.2.1. In the case of simple pigmented feeds, mixed feeds, or in cases of doubt, use the two-dimensional thin-layer chromatographic method described in 8.7.2.2.

8.7.2.1 One-dimensional thin-layer chromatography

Trace a straight line on a plate (6.7) to divide it into two equal parts. Apply on each part, 20 mm from the lower edge and at intervals of 15 mm, the volumes indicated below of the standard aflatoxin B_1 solution and the extract :

— 25 µl of the standard aflatoxin B_1 solution (5.14);

— a volume of the extract obtained in 8.4.2 containing approximately 2,5 ng of aflatoxin B_1;

— 25 µl of the standard aflatoxin B_1 solution (5.14) and, superimposed on it, a volume of the extract obtained in 8.4.2 containing approximately 2,5 ng of aflatoxin B_1.

Apply to one of the two halves of the plate, superimposed on the spots previously applied, 1 to 2 µl of the trifluoroacetic acid (5.11). Dry in a stream of air at ambient temperature.

Develop the chromatogram, in the dark, using one of the developing solvents (5.6). The choice of the solvent shall be made beforehand. The solvent system shall ensure that the aflatoxin B_1-hemiacetal (aflatoxin B_{2a}) is clearly separated from interfering substances. The solvent front shall travel about 120 mm.

Allow the solvents to evaporate in the dark, and then spray sulfuric acid (5.13) on to the part of the plate not previously treated with the trifluoroacetic acid. Examine the plate under ultraviolet light.

The identity of aflatoxin B_1 is confirmed if

a) the R_f value of the aflatoxin B_1 derivative originating from the extract corresponds with that from the standard solution;

b) the aflatoxin B_1 derivative originating from the standard solution, superimposed on the extract, has a fluorescence more intense than the aflatoxin B_1 derivative originating from the extract.

Since fluorescent spots from the extract, having the same R_f value as the aflatoxin B_1-hemiacetal, might lead to a false

positive interpretation of the chromatogram, their presence should be checked on the part of the plate treated with sulfuric acid.

In cases of doubt, confirmation by two-dimensional thin-layer chromatography (8.7.2.2) shall be used.

8.7.2.2 Two-dimensional thin-layer chromatography (see figure 3)

8.7.2.2.1 Application of the solutions

Trace two straight lines on a plate (6.7), parallel to two contiguous sides (60 mm from each side), to establish the limit of migration of the solvent fronts. Apply the following solutions on the plate using capillary pipettes or microsyringes :

— at point A : a volume of purified extract from the sample, obtained in 8.4.2, containing about 2,5 ng of aflatoxin B_1, and a drop (1 to 2 µl) of the trifluoroacetic acid (5.11);

— at points B and C : 25 µl of the standard aflatoxin B_1 solution (5.14), and a drop of the trifluoroacetic acid (5.11).

Dry in a stream of air at ambient temperature.

8.7.2.2.2 Development

Develop the chromatogram in direction I, in the dark, using the developing solvent (5.6.2) (1 cm layer in an unsaturated tank) until the solvent front reaches the limit line. Remove the plate from the tank and allow to dry, in the dark, at ambient temperature, for 5 min.

Then develop the chromatogram in direction II, in the dark, using the developing solvent (5.6.1) (1 cm layer in an unsaturated tank) until the solvent front reaches the limit line. Remove the plate from the tank and allow to dry at ambient temperature.

8.7.2.2.3 Interpretation of the chromatogram

Examine the chromatogram under ultraviolet light from the lamp (6.8) and check for the following features :

a) appearance of a blue fluorescent spot of aflatoxin B_1-hemiacetal, and sometimes a weak blue fluorescent spot of aflatoxin B_1 which has not reacted with the trifluoroacetic acid, originating from the standard solution applied at point C (migration in direction I) and from the standard solution applied at point B (migration in direction II);

b) appearance of spots similar to those described in a), originating from the sample extract applied at point A. The position of these spots is defined by those originating from the standard solution applied at points B and C. The intensities of fluorescence of the aflatoxin B_1-hemiacetal spots originating from the extract and from the standard solution applied at points B and C should be comparable.

8.8 Number of determinations

Carry out two determinations on the same test sample.

9 Expression of results

9.1 Method of calculation and formulae

9.1.1 Visual measurements

The aflatoxin B_1 content, expressed in micrograms per kilogram of sample, is equal to

$$\frac{C \times V_1 \times V_3}{m \times V_2}$$

where

C is the concentration, in micrograms of aflatoxin B_1 per millilitre, of the standard solution (5.14) (approximately 0,1 µg/ml);

m is the mass, in grams, of the test portion corresponding to the volume of extract subjected to column clean-up (10,0 g);

V_1 is the final volume of the extract, in microlitres, taking into account any dilution that was necessary;

V_2 and V_3 are, respectively, the volumes, in microlitres, of the extract and of the standard aflatoxin B_1 solution (5.14), applied on the plate, having similar intensities of fluorescence.

9.1.2 Fluorodensitometric measurements

The aflatoxin B_1 content, expressed in micrograms per kilogram of sample, is equal to

$$\frac{m_1 \times V_1}{m \times V_2}$$

where

m is the mass, in grams, of the test portion corresponding to the volume of extract subjected to column clean-up (10,0 g);

m_1 is the mass, in nanograms, of aflatoxin B_1 in the extract spot (taking into account the volume V_2), deduced from the measurements;

V_1 is the final volume of the extract, in microlitres, taking into account any dilution that was necessary;

V_2 is the volume, in microlitres, of extract applied on the plate (10 or 20 µl).

9.2 Precision

Three round-robin trials, two of which were carried out at the international level (Nos. 1 and 2), on mixed feeding stuffs (method B) gave the results indicated in the table.

The 11 laboratories participating in trial 2 also analysed the sample by method A, its composition being suitable, and obtained results very similar to those when using method B, by visual or fluorodensitometric measurement.

10 Test report

The test report shall show the method used (A or B), the method of determination (visual or fluorodensitometric measurement) and the results obtained. It shall also mention all operating conditions not specified in this International Standard, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all the information necessary for the complete identification of the sample.

Parameter	Sample		
	1	2	3
Number of laboratories	23	11	13
Mean	162,7 µg/kg	25,4 µg/kg	13,4 µg/kg
Standard deviation of repeatability (s_r)	16,9 µg/kg	2,7 µg/kg	1,7 µg/kg
Coefficient of variation of repeatability	10 %	11 %	13 %
Repeatability (2,83 s_r)	47,8 µg/kg	7,6 µg/kg	4,8 µg/kg
Standard deviation of reproducibility (s_R)	45,2 µg/kg	6,8 µg/kg	4,0 µg/kg
Coefficient of variation of reproducibility	28 %	27 %	30 %
Reproducibility (2,83 s_R)	128,0 µg/kg	19,2 µg/kg	11,3 µg/kg

Dimensions in millimetres

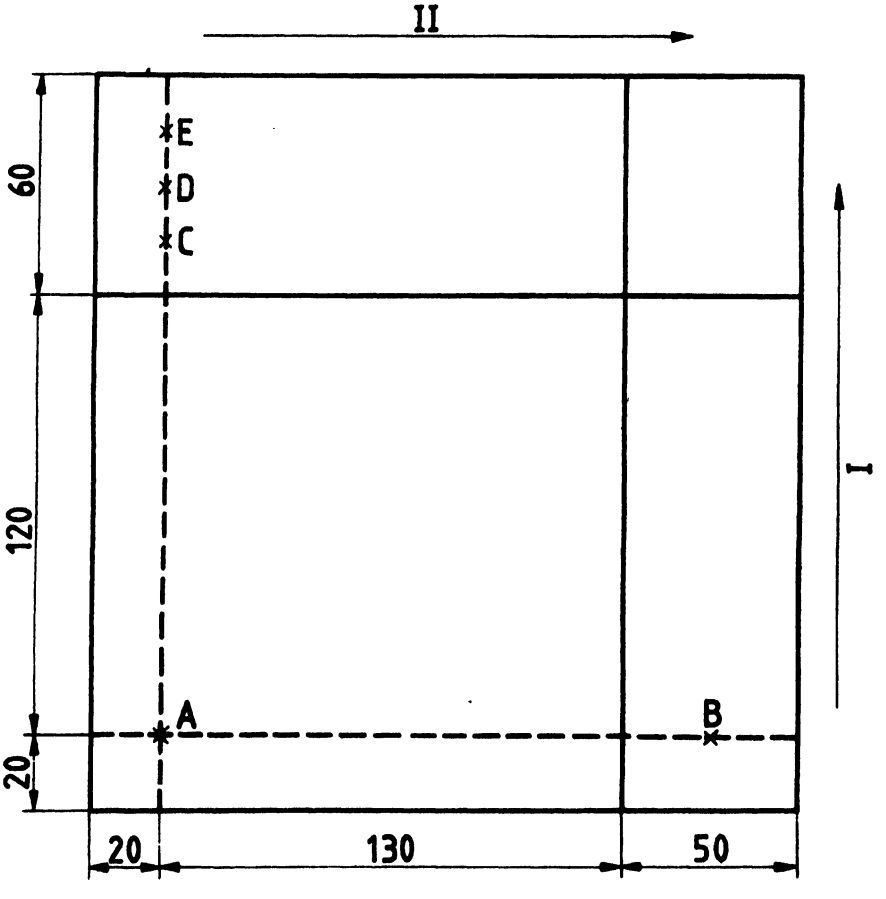


Figure 1 — Application of solutions

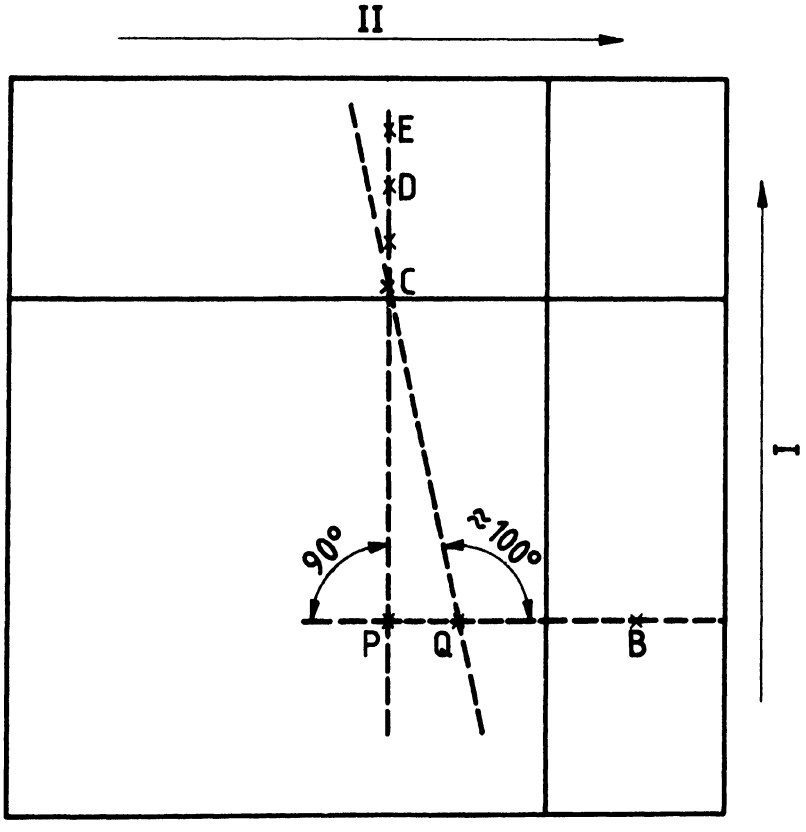


Figure 2 — Interpretation of the chromatogram

Dimensions in millimetres

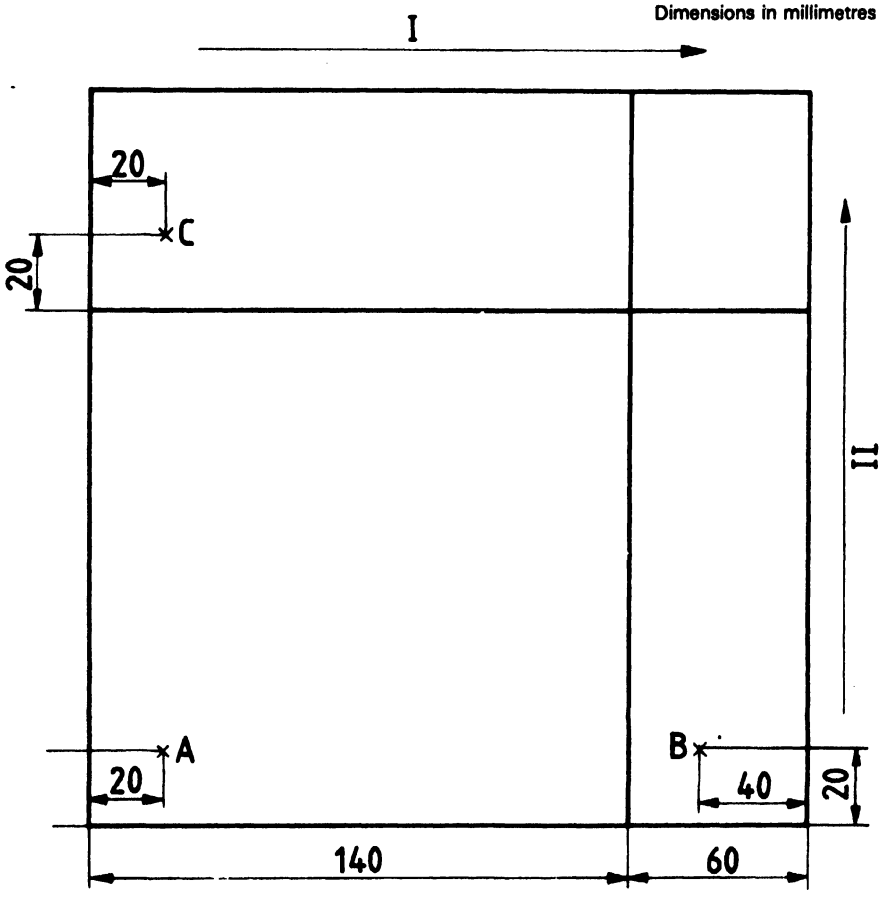


Figure 3 — Confirmation test

Reprography Unit, BIS, New Delhi, India

Bureau of Indian Standard

BIS is a statutory institution established under the *Bureau of Indian Standards Act*, 1986 to promote harmonious development of the activities of standardization, marking and quality certification of goods and attending to connected matters in the country.

Copyright

BIS has the copyright of all its publications. No part of these publications may be reproduced in any form without the prior permission in writting of BIS. This does not preclude the free use, in the course of implementing the standard, of necessary details, such as symbols and sizes, type or grade designation. Enquiries relating to copyright be addressed to the Director (Publications), BIS.

Revision of Indian Standards

Indian Standards are reviewed periodically and revised, when necessary and amendments, if any, are issued from time to time. Users of Indian Standards should ascertain that they are in possession of the latest amendments or edition. Comments on this Indian Standard may be sent to BIS giving the following reference:

Doc : No FAD 5 (163)

Amendments Issued Since Publication

Amend No.	Date of Issue	Text Affected

BUREAU OF INDIAN STANDARDS

Headquarters:

Manak Bhavan, 9 Bahadur Shah Zafar Marg, New Delhi 110002
Telephones : 331 01 31, 331 13 75

Telegrams : Manaksanstha
(Common to all Offices)

Regional Offices :	Telephone
Central : Manak Bhavan, 9 Bahadur Shah Zafar Marg, NEW DELHI 110002	331 01 31, 331 13 75
Eastern : 1/14 C. I. T. Scheme VII M, V. I. P. Road, Maniktola, CALCUTTA 700054	37 84 99, 37 85 61, 37 86 26, 37 85 62
Northern : SCO 445-446, Sector 35-C, CHANDIGARH 160036	53 38 43, 53 16 40, 53 23 84
Southern : C. I. T. Campus, IV Cross Road, MADRAS 600113	41 24 42, 41 25 19, 41 23 15, 41 29 16,
Western : Manakalaya, E9 MIDC, Marol, Andheri (East), BOMBAY 400093	632 92 95, 63 27 80, 632 78 92

Branches : AHMADABAD, BANGALORE, BHOPAL, BHUBANESHWAR, COIMBATORE FARIDABAD, GHAZIABAD, GUWAHATI, HYDERABAD, JAIPUR, KANPUR, LUCKNOW, PATNA, THIRUVANANTHAPURAM.

Printed at Printwell Printers, Aligarh, India